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14. ABSTRACT p53 is regulated at the multiple levels. We report here that p53 in multiple lines of human cancer cells is down-regulated by cardiac glycoside drugs, digoxin or ouabain, the potent inhibitors of Na ⁺ /K ⁺ -ATPase. These drugs reduced the basal levels of p53 protein at nanomolar concentrations in a dose-, time- and cancer cell line-dependent manner, but independent of p53 status of wild type (wt) or mutant. The drugs also reduced the levels of p53 induced by its activators as well as p53 transfected into human cancer cells, regardless of its status. Interestingly, the drugs had no effect on endogenous p53 in two immortalized human cell lines. Mechanistically, p53 reduction is because reduced protein synthesis. Finally, p53 reduction appears to be triggered by activation of Src/MAPK signaling pathways upon drug binding to the Na ⁺ /K ⁺ -ATPase and can be completely blocked by the inhibitors of Src or MEK. This is the first report that cardiac glycoside drugs, by initiating the Src/MAPK signaling pathways, reduce the p53 levels via inhibition of p53 protein synthesis. The drugs may be useful in the treatment of human cancers with a gain-of-function p53 mutation.					
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Final report for W81XWH-08-1-0539

Chemoprevention by elimination of cancer-prone, mutant p53-containing breast cells

Yi Sun, Ph.D.

Introduction:

The purpose of this study is to test our working hypothesis that a gene product exists in human cells which is synthetic lethal to p53 mutation upon inhibition. A natural product compound can be identified that inhibits this gene product to selectively kill breast cancer cells with p53 mutation, while has minimal toxic effect on normal breast cells. The natural product(s) identified here, with known little toxicity, could be used as a chemoprevention agent that kills p53 mutation-bearing breast cancer cells at the early stage via the synthetic lethal mechanism, thus preventing its full growth to a solid tumor.

Body:

1). Screen procedure (performed at NIH Chemical Genomic Center)

- a) Seed cells (H1299-neo and H1299-ts) at 1536 well plates
- b) incubate all cells at 37oC for 8 hr
- c) add nature products (5 concentrations: 0.1104, 0.552, 2.76, 13.8, 69 ug/ml)\
- d) separate cell plates and incubate at 37oC or 32oC for 24 hrs
- e) ATPlite assay to test the cell viability

2) Lead identification

After screening ~6,000 natural compounds, we identified few positive hits that selectively kill H1299-TS cells at 37oC (mutant p53 confirmation) with limited killing in all other three conditions (H1299-ts at 32oC, H1299-neo at both 32oC and 37oC). We focused on one which was identified multiple times with the same natural product strain.

NMIX00007760	Umich2-031-082	Strain 06-307-4N, MEOH extraction (#21)
NMIX00007762	Umich2-031-081	Strain 06-307-4N*, Acetone extraction (#22)
NMIX00007743	Umich2-030-269	Strain 06-307-4N*, EtoAC extraction (#23)
NMIX00007741	Umich2-030-268	Strain 06-307-4N, Cells+MeOH extraction (#24)

3. Follow-up study--#1.

We went on and collaborated with Dr. David Sherman at the University of Michigan who deposited these natural products at NIH-CGC). We focused on fraction #23 and found that this fraction was indeed active. Carl Vrevang at Dr. Sherman's group then did a large preparation, followed by fractionation. We identified an active fraction (Fraction D), among several inactive fractions (Fractions A-C), that killed cancer cells more selectively than normal cells, but it did not selectively kill cancer cells with mutant p53. For example, the IC50 for breast cancer lines, harboring a mutant p53, MDA-MB231 and SK-BR3 were 1.21 and 0.86 ug/ml, respectively. The IC50 for H1299 lung cancer cells without p53 was 0.77 ug/ml, whereas for normal lung fibroblast cells, IC50 was 2.58 ug/ml. We repeated several times with other extraction methods (#21, #22) and

were not able to identify an active fraction that was able to selectively kill cancer cells with a mutant p53.

4. Follow-up study #2.

During the validation of small molecule hits of the synthetic lethal screen, we serendipitously found that cardiac glycoside drugs, digoxin and ouabain, reduced the p53 levels in a time- and dose-dependent manner in some sensitive cancer cell lines, including breast cancer lines. Our follow-up study with this finding led to a publication in Cancer Res (69: 6556-64, 2009).

Key research accomplishments:

- 1) Identification of few extracts from a particular sea-weed that selectively inhibited the growth of human cancer cells harboring a mutant p53. However, follow-up fractionation identified a particular fraction that selectively inhibited the growth of cancer cells, regardless of p53 status.
- 2) Identification of cardiac glycosides that inhibited p53 synthesis via Src and MAPK pathways. The compounds may have utility for the treatment of human cancer harboring a gain-of-function p53 mutant.

Reportable outcomes:

A publication in Cancer Research (attached)

Cardiac glycosides reduce the p53 levels via inhibiting p53 protein synthesis: Rescued by Src and MAPK inhibitors

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Noel Southall³, Shaomeng Wang⁴, Menghang Xia³, Christopher P. Austin³, Wei Zheng³,
Zijian Xie², and Yi Sun^{1#}

Cancer Research 69: 6556-64, 2009

Abstract

p53 is regulated at the multiple levels. We report here that p53 in multiple lines of human cancer cells is down-regulated by cardiac glycoside drugs, digoxin or ouabain, the potent inhibitors of Na⁺/K⁺-ATPase. These drugs reduced the basal levels of p53 protein at nanomolar concentrations in a dose-, time- and cancer cell line-dependent manner, but independent of p53 status of wild type (wt) or mutant. The drugs also reduced the levels of p53 induced by its activators as well as p53 transfected into human cancer cells, regardless of its status. Interestingly, the drugs had no effect on endogenous p53 in two immortalized human cell lines. Mechanistically, p53 reduction did not occur at the mRNA levels, but at the protein levels, as a result of reduced protein synthesis rather than enhanced degradation. The cellular sensitivity to drug-induced p53 reduction was not associated with the levels of α subunits of Na⁺/K⁺-ATPase in different cell lines. While lowering extracellular K⁺ did not reduce p53 as did ouabain and digoxin, it did potentiate both digoxin and ouabain-induced p53 reduction in sensitive lines. Finally, p53 reduction appears to be triggered by activation of Src/MAPK signaling pathways upon drug binding to the Na⁺/K⁺-ATPase and can be completely blocked by the inhibitors

of Src or MEK. This is the first report that cardiac glycoside drugs, by initiating the Src/MAPK signaling pathways, reduce the p53 levels via inhibition of p53 protein synthesis. The drugs may be useful in the treatment of human cancers with a gain-of-function p53 mutation.

Conclusion:

- 1) Some sea weed extracts have activity in selective growth inhibition of human cancer cells with a mutant p53 via synthetic lethal mechanism. However, further fractionations were proved to be difficult in identification of active components.
- 2) Cardiac glycosides, the drugs used in clinic for treatment of heart failure were found to inhibit p53 synthesis via Src and MAPK pathways. The compounds may have utility for the treatment of human cancer harboring a gain-of-function p53 mutant.

References:

Wang, Z.^{*}, Zheng, M.^{*}, Li, Z., Li, R., Jia, L., Xiong, X., Southall, N., Wang, S., Xia, M., Austin, C.P., Zheng, W., Xie, Z. and **Sun, Y.**: Cardiac glycosides inhibit p53 synthesis by a mechanism relieved by Src or MAPK inhibition. Cancer Res. 69: 6556-6564, 2009.

Cardiac Glycosides Inhibit p53 Synthesis by a Mechanism Relieved by Src or MAPK Inhibition

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Abstract

p53 is regulated at multiple levels. We report here that p53, in multiple lines of human cancer cells, is down-regulated by cardiac glycoside drugs digoxin and ouabain, potent inhibitors of Na⁺/K⁺-ATPase. These drugs reduced the basal levels of p53 protein at nanomolar concentrations in a dose-, time-, and cancer cell line-dependent manner, but independent of p53 status of wild-type or mutant. The drugs also reduced the levels of p53 induced by its activators as well as p53 transfected into human cancer cells, regardless of its status. Interestingly, the drugs had no effect on endogenous p53 in two immortalized human cell lines. Mechanistically, p53 reduction occurred not at the mRNA levels but at the protein levels, as a result of reduced protein synthesis rather than enhanced degradation. The cellular sensitivity to drug-induced p53 reduction was not associated with the levels of α subunits of Na⁺/K⁺-ATPase in different cell lines. Although lowering extracellular K⁺ did not reduce p53 as did ouabain and digoxin, it did potentiate both digoxin- and ouabain-induced p53 reduction in sensitive lines. Finally, p53 reduction seems to be triggered by activation of Src/mitogen-activated protein kinase (MAPK) signaling pathways upon drug binding to the Na⁺/K⁺-ATPase and can be completely blocked by the inhibitors of Src or MAP/ERK kinase. This is the first report that cardiac glycoside drugs, by initiating the Src/MAPK signaling pathways, reduce the p53 levels via inhibition of p53 protein synthesis. The drugs may be useful in the treatment of human cancers with a gain-of-function p53 mutation. [Cancer Res 2009;69(16):6556–64]

Introduction

p53 prevents tumor formation through transcription-dependent and transcription-independent mechanisms. Transcription-dependent mechanism is mainly mediated by p53 up-regulation of its downstream targets. Upon activation by a variety of stimuli,

p53 induces the expression of proarrest genes, such as p21, Gadd45, or 14-3-3 σ to induce growth arrest or of proapoptotic genes, such as PUMA, PIG-3, or DR5 to induce apoptosis (1). Through a direct binding to mitochondria and modulating BH3 family proapoptotic proteins, such as Bax, p53 can also regulate apoptosis in a transcription-independent manner (2, 3). Thus, p53 acts as a guardian of the genome by inducing growth arrest to allow cells to repair the damage or apoptosis, if the damage is too severe and irreparable.

Because p53 plays a pivotal role in controlling abnormal cell growth and is inactivated by point mutations in >50% human cancers, p53 has been a central target for mechanism-driven cancer drug discovery (4, 5). Significant progress has been made in past decade, leading to identification and characterization of several unique classes of small molecules that modulate p53 (6, 7). They can be categorized as follows: (a) the molecules that restore wild-type (wt) p53 from a mutant conformation, which include CP-31398 (8), PRIMA-1 (9), and ellipticine (10); (b) the molecules that target Mdm2 to reactivate p53, including Mdm2 E3 ubiquitin ligase inhibitor, HLI98 (11), and inhibitors that disrupt Mdm2-p53 binding, such as Nutlin (12), RITA (13), and MI-219 and its analogues (14–16); (c) the molecules that inhibit wt p53, including pifithrin- α (17), and pifithrin- μ (18); and (d) the molecules that selectively degrade mutant p53, including Hsp90-active agents such as geldanamycin (19) and histone deacetylase inhibitors, such as trichostatin (20).

During the screening for small molecules that selectively kill mutant-p53 containing cancer cells via a synthetic lethal mechanism (21), we serendipitously found that cardiac glycoside drugs, digoxin and ouabain, reduced the p53 levels in a time- and dose-dependent manner in sensitive cancer cell lines. The drug sensitivity to p53 reduction is cancer cell line-dependent, but independent of p53 status of a wt or mutants. Importantly, the drugs are completely inactive in reducing wt p53 in normal “immortalized” cells. Mechanistically, the drug-induced p53 decrease occurred not at the mRNA levels, but at the protein levels, as a result of reduced synthesis, rather than enhanced degradation. The drug-induced p53 reduction can be rescued by the inhibitors of Src and MAP/ERK kinase (MEK), suggesting an involvement of Src/mitogen-activated protein kinase (MAPK) signaling pathways, initiated upon the drug binding to Na⁺/K⁺-ATPase. Our study revealed a novel mechanism by which activation of Src/MAPK kinase pathway could eventually lead to p53 elimination by inhibiting p53 protein synthesis.

Materials and Methods

Cell culture and drug treatment. All cell lines used in this study, except those mentioned below, were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum. H1355, HCT116, and MRC5 were maintained in

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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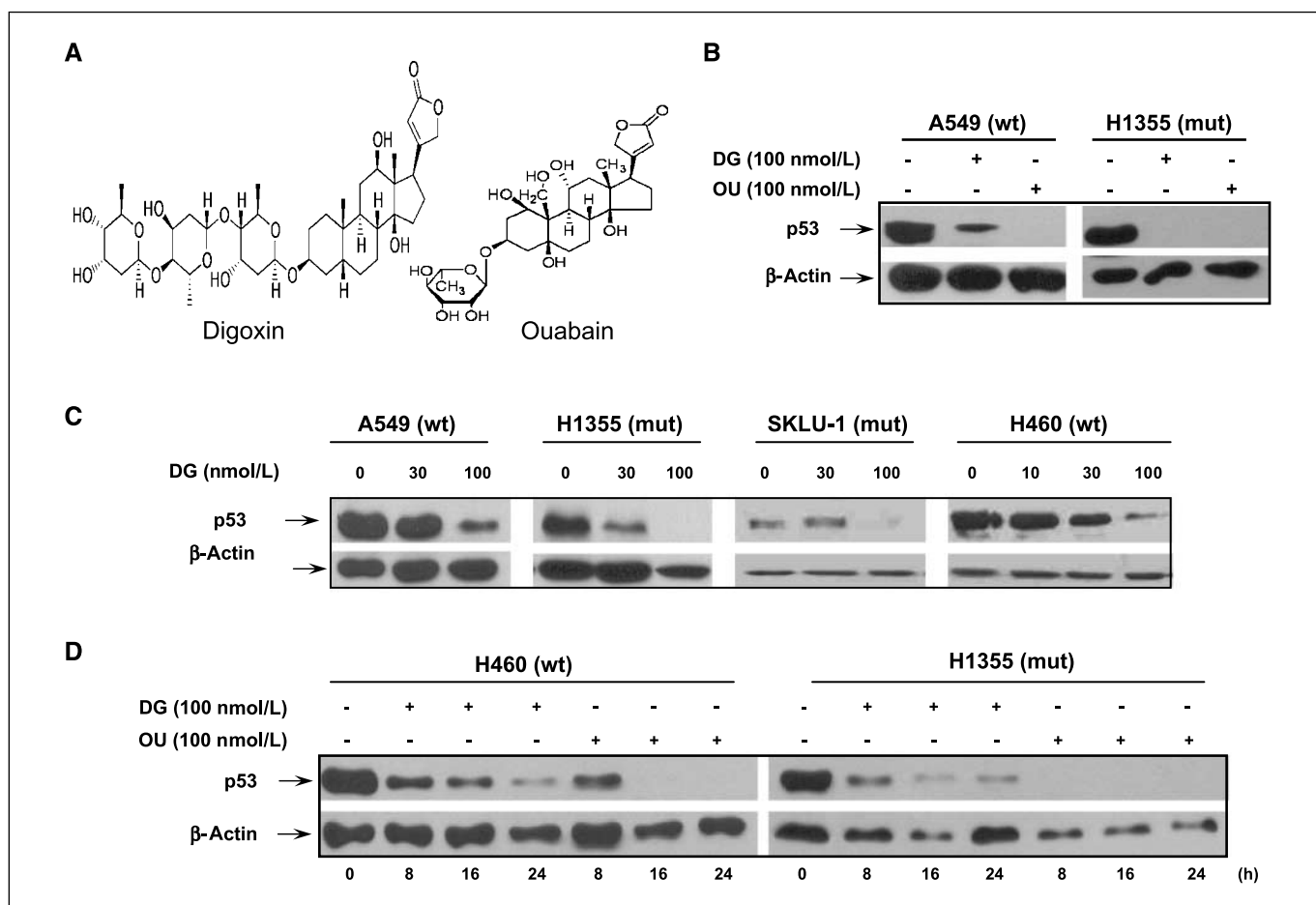


Figure 1. Cardiac glycosides digoxin (DG) and ouabain (OU) reduce basal p53 levels in lung cancer cell lines: dose and time dependent. **A**, structure of digoxin and ouabain. **B**, digoxin or ouabain reduces p53 levels: A549 and H1355 lung cancer cells were treated with digoxin or ouabain at 100 nmol/L for 24 h and subjected to Western blotting. **C**, dose-dependent reduction of p53 levels by digoxin: Four lung cancer lines were treated with digoxin at 30 or 100 nmol/L for 24 h and subjected to Western blotting. **D**, time-dependent reduction of p53 levels by digoxin or ouabain: H460 and H1355 cells were treated with digoxin or ouabain at 100 nmol/L for indicated periods of time, followed by Western blotting.

RPMI 1640 and McCoy's 5A medium, respectively, containing 10% serum. NL20 cells were cultured in Ham's F12 medium as described (22). For drug treatment, subconfluent cells were treated with digoxin or ouabain alone or in combination of PP2, PD098059, or LY294002 (Sigma).

For culturing cells in low K^+ conditions, A549 or H1355 cells were cultured in 10% DMEM until the cell densities reached 80%. The medium was replaced by K^+ -free DMEM supplemented with normal K^+ concentration (5 mmol/L) or low K^+ concentrations (1 or 0.3 mmol/L). Na^+ was added to the low K^+ medium to maintain the equal ion concentrations (23).

Western blotting analysis. The assay was done as described previously (16). The antibodies used are p53 (Calbiochem; 1:1,000); p21 (BD; 1:1,000); Mdm2 (Calbiochem; 1:500); FAK, Src, or tubulin (Santa Cruz Biotechnology; 1:1,000); pSrc at Y418 (Invitrogen; 1:1,000); pERK1/2 (Cell Signaling; 1:1,000); Na^+/K^+ -ATPase $\alpha 1$ (Developmental Studies Hybridoma Bank at the University of Iowa; 1:500); Na^+/K^+ -ATPase $\alpha 3$ (Affinity BioReagents; 1:1,000); and β -actin (Sigma; 1:5,000).

Transfection and infection. H1299 cells were plated into six-well plate at 2×10^5 cells per well and transfected the following day with 1 μ g of plasmid expressing either p53-wt or p53-mutants for 24 h before drug treatment. For the infection of sh-RNA targeting FAK viruses (gift from Jun-Lin Guan, University of Michigan, Ann Arbor, MI), a total of 10^9 plaque-forming unit viruses were infected into 2×10^6 A549 or H1355 cells per 100-mm dish for 72 h. Cells were split, followed by exposure to drugs next day for 24 h. To silence endogenous wt p53, A549 or H460 cells were infected with a lentivirus-based siRNA construct, LT-p53-siRNA, as described (16).

ATPlite growth assay and IC_{50} determination. Cells were seeded in 96-well white plates and treated with the drugs with a range of indicated concentrations for 24 h. The viability of the cells was then measured using a one-step ATPlite kit (Perkin-Elmer). The results were calculated and plotted in Prism 4.0 (Graphpad) to generate IC_{50} curves (22).

Quantitative reverse transcription-PCR. Total RNA was isolated from cells after drug treatment, using a Trizol kit (Invitrogen), and subjected to quantitative reverse transcription-PCR (RT-PCR) analysis, using QuantiTect SYBR green RT-PCR kit (Qiagen). Briefly, 50 μ L reaction mixture were used for each reaction, which contained $2 \times$ QuantiTect SYBR Green RT-PCR Master Mix, 0.5 μ L QuantiTect RT mix, 0.5 μ mol/L primer mix, and 0.5 μ g RNA. Cycling program was set as the following: 50°C 30 min for RT, 95°C 15 min for the PCR initial activation and 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The sequences of p53 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are as follows: hu-p53 F1:TCTGTGACTTGCACGTAC; hu-p53 R1: ATTTCCTTCCACTCGGAT. GAPDH-F1: GTTGCCATCAATGACCCCTT; GAPDH-R1: AGAGGCAGGGATGATGTTCT.

^{35}S -Met metabolic labeling. Subconfluent cells were treated with the drugs for various time points with last hour in methionine and cysteine-free DMEM, containing 5% dialyzed FCS and 50 μ mol/L MG132. Cells were then labeled with 100 μ Ci/mL of [^{35}S]-methionine (MP Biochemicals) for 5 min, followed by immunoprecipitation with anti-p53 antibody (Santa Cruz).

Immunoprecipitates, along with whole cell extract, were then subjected to SDS-PAGE and autoradiography. The steady-state levels of p53 were measured by immunoprecipitated, followed by Western blotting, along with the detection of total cellular proteins by Coomassie staining.

Results

Cardiac glycosides reduced basal levels of p53 in lung cancer cell lines. During our confirmation of candidates identified from a chemical library screen for selective killing of cancer cells with mutant p53 via synthetic lethal mechanism (21), we serendipitously found that cardiac glycosides, including digoxin and ouabain (Fig. 1A for structure), are able to reduce the p53 levels. As shown in Fig. 1B, digoxin or ouabain at the nanomolar concentrations reduced or eliminated the basal levels of p53 in two lung cancer lines, A549 with a wt p53 and H1355 with a mutant p53, with ouabain being more potent. Digoxin-induced p53 reduction or elimination was the dose-dependent, but the p53 status independent in four lung cancer cell lines with either wt or mutant p53 status (Fig. 1C). Furthermore, p53 reduction is treatment-time dependent, starting to occur at 8 hours with a complete elimination seen by ouabain at 16 hours after drug exposure (Fig. 1D). Finally, cell line-dependent, but p53 status-independent, p53 reduction or elimination by digoxin or ouabain can be extended to multiple human cancer cell lines. These include colon cancer lines, DLD1, but not HCT116, nor HT29; breast cancer lines, MCF7, but not MDA-MB231; all three head and neck squamous carcinoma lines, but not three glioblastoma lines tested (Supplementary Materials; Supplementary Fig. S1). Digoxigenin, another cardiac glycoside was also active, but 10-fold less potent in reducing p53 levels in multiple human cancer lines (data not

shown). Our results clearly showed that p53 levels in multiple human cancer cell lines are subjected to reduction or elimination by cardiac glycosides, digoxin or ouabain, in a cell line-dependent, but p53 status-independent manner.

Cardiac glycosides blocked p53 induction and p53 up-regulation of its target genes by p53 activators. We next determined if digoxin or ouabain also reduced the levels of induced p53 and p53 transactivation of its target genes by known p53 activators. Three lung cancer cell lines were treated for 24 hours with MI-219, an Mdm2 inhibitor, which activates p53 by disrupting Mdm2-p53 binding (14) or etoposide, a DNA damaging agent, known to activate p53 in wt p53-containing lung cancer cells (16), alone or in combination with digoxin or ouabain, respectively. As shown in Fig. 2A, MI-219 or etoposide treatment induced p53 levels as well as p53 target proteins, p21 or Mdm2 (by MI-219 only), in two wt p53-containing lines, A549 and H460, but not in mutant p53-containing line, H1355 (*lanes 1–3*). Combinational treatment with either digoxin or ouabain reduced the induced levels of p53 and completely eliminated p53 induction of its targets, p21 and Mdm2 in A549 and H460 cells (*lanes 4–9*). The mutant p53 in H1355 was not subjected to induction by two p53 activators, but was completely eliminated in combinational treatment (*bottom*).

p53 can be stabilized by hypoxia in wt p53 containing MCF7 cells by chemical hypoxic agents, cobalt chloride (CoCl₂), and desferrioxamine (24). We next determined if hypoxia-stabilized p53 is also subjected to reduction by digoxin or ouabain. As shown in Fig. 2B, a 24-hour treatment of CoCl₂ stabilized p53 up to 2-fold in MCF7 cells. This stabilized p53 was reduced and eliminated by simultaneous treatment of digoxin or ouabain in a dose-dependent manner up to 300 nmol/L (*lanes 3–8*). Along with p53 reduction, two p53 target proteins, p21 and Mdm2, which were detectable at

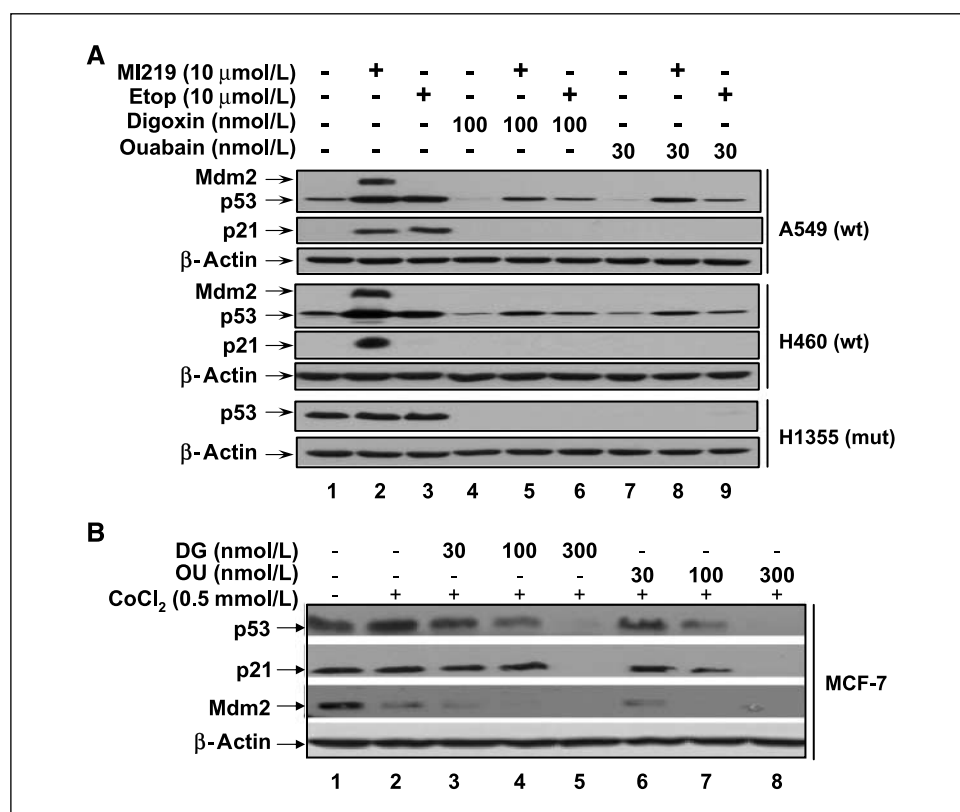


Figure 2. Digoxin or ouabain reduces the levels of induced or stabilized p53 and blocks p53 transactivation of its targets. **A**, reduction of induced p53: Lung cancer cells with either wt p53 or mutant p53 were treated with p53 activator MI219 or etoposide (*etop*) alone or in combination of digoxin or ouabain. **B**, reduction of stabilized p53: MCF7 cells were treated with CoCl₂ (0.5 mmol/L) alone or in combination with digoxin or ouabain for 24 h. Cell lysates were prepared for Western blotting.

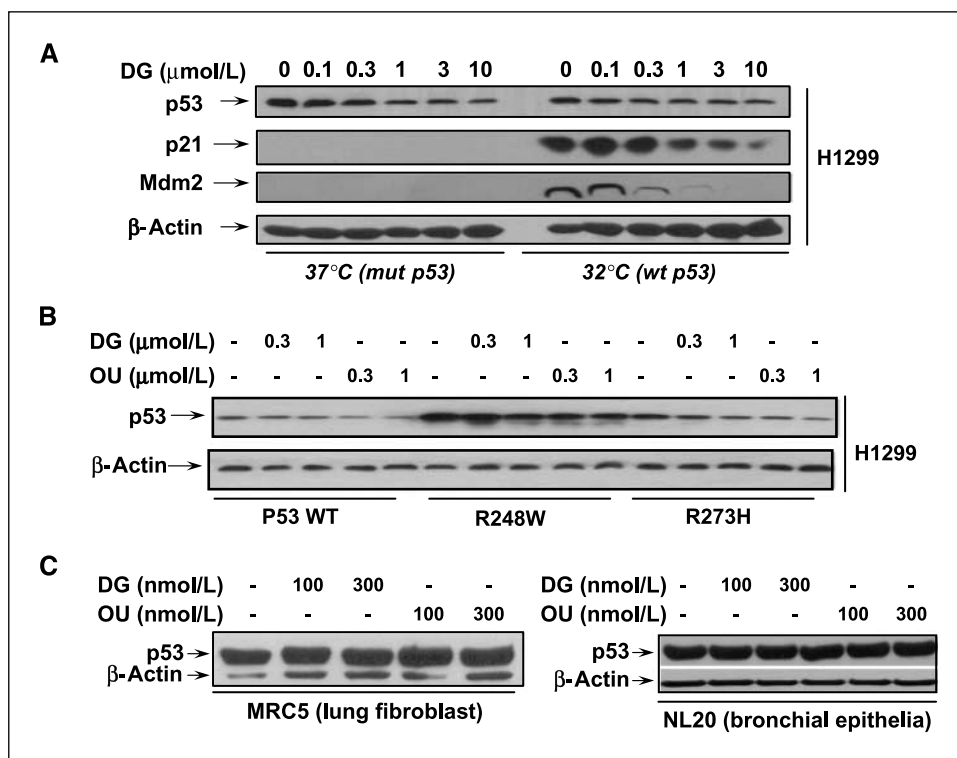


Figure 3. Digoxin or ouabain selectively reduces the p53 levels in cancer cells, but not in immortalized normal cells. **A**, p53 status-independent reduction of stably overexpressed p53: A stable clone of H1299 lung cancer cells (endogenous p53-null), overexpressing a temperature-sensitive p53 mutant (H1299-p53^{ts}-A138V), was treated with various concentrations of digoxin as indicated while grown at 37°C (adapting a mutant p53 conformation) or 32°C (adapting a wt p53 conformation). The levels of p53 and its two target proteins, p21 and Mdm2, were measured by Western blotting. **B**, p53 status-independent reduction of transiently overexpressed p53: p53-null H1299 cells were transiently transfected with wt p53 or two p53 mutants most frequently found in human cancer (p53-R248W and p53-R273H). Cells were subsequently treated with the drugs for 24 h, followed by Western blotting. **C**, lack of p53 reduction by digoxin or ouabain in two immortalized lung-derived cells: Lung fibroblast MRC5 and bronchial epithelial NL20 cells were treated with digoxin or ouabain for 24 h, followed by Western blotting.

the basal levels due to a relatively higher level of p53, were also reduced and eliminated. Taken together, these results indicate that both cardiac glycosides also reduced the levels of p53 and its target proteins upon p53 activation and stabilization. The results further suggest that drug-induced p53 reduction may be Mdm2 independent because Mdm2 is free from p53 binding upon MI-219 treatment (14).

Cardiac glycosides reduced the levels of transfected p53 in cancer cells, but had no effect on endogenous wt p53 in normal cells. We next determined if p53 upon overexpression in a p53-null H1299 cells would also be subjected to digoxin or ouabain reduction. H1299 cells overexpressing a temperature sensitive p53 mutant (codon 138; ref. 25) were treated with digoxin for 24 hours at 37°C (p53 adopts a mutant conformation) or 32°C (wt conformation). As shown in Fig. 3A, digoxin treatment caused a dose-dependent reduction of overexpressed p53, regardless of p53 status. Upon p53 reduction, p53 target genes, p21 and Mdm2, induced at 32°C when p53 is in a wt status, were also reduced accordingly. Likewise, digoxin reduced the levels of p53 transiently transfected into H1299 cells regardless of the p53 status of wt or two mutants, R248W and R273H, most frequently found in human cancer (Fig. 3B). However, the potency of drugs against overexpressed p53 was much reduced with much high drug doses up to 10 μmol/L to achieve a moderate effect. Interestingly, digoxin or ouabain failed to reduce the endogenous wt p53 highly expressed in two immortalized lung-derived cell lines, MRC5 lung fibroblast and NL20 bronchial epithelia (Fig. 3C). These results indicated a tumor

cell line selective and p53 status-independent reduction of p53 by digoxin or ouabain.

Dissociation between drug cytotoxicity and drug-induced p53 reduction. To exclude the possibility that p53 reduction is the consequence of drug-induced cytotoxicity, we measured the IC₅₀ values of a panel of cancer cell lines, either sensitive or resistant to drug-induced p53 reduction. As shown in Supplementary Fig. S2, digoxin or ouabain was quite potent growth inhibitor with an IC₅₀ ranging from 50 to 100 nmol/L in the majority of lines tested. However, the drug cytotoxicity seems not to be associated with cellular sensitivity to drug-induced p53 reduction. For examples, two sensitive lines, H1355 and DLD1, had similar IC₅₀ values to two resistant lines, HCT116 and HT29, whereas MCF7 cells are rather resistant to digoxin cytotoxicity (Supplementary Fig. S2A), but sensitive to digoxin-induced p53 reduction (Supplementary Fig. S1). The morphologic appearances of A549 and H1355 after drug treatment for different periods of time were shown in Supplementary Fig. S3, which showed a moderate cytotoxicity only at 24 hours, particularly in H1355 cells. Furthermore, because two wt p53-containing lines, A549 and H460 seemed to be the most sensitivity to drug cytotoxicity with an IC₅₀ of 20 to 40 nmol/L, we determined if the cytotoxicity is p53 dependent. As shown in Supplementary Fig. S2B to D, siRNA silencing of p53 did not change drug cytotoxicity, indicating that cytotoxicity is not mediated through, nor associated with wt p53.

Cardiac glycosides-induced p53 reduction does not occur at the mRNA levels, but at the protein levels. p53 regulation by digoxin or ouabain could occur at the levels of transcription,

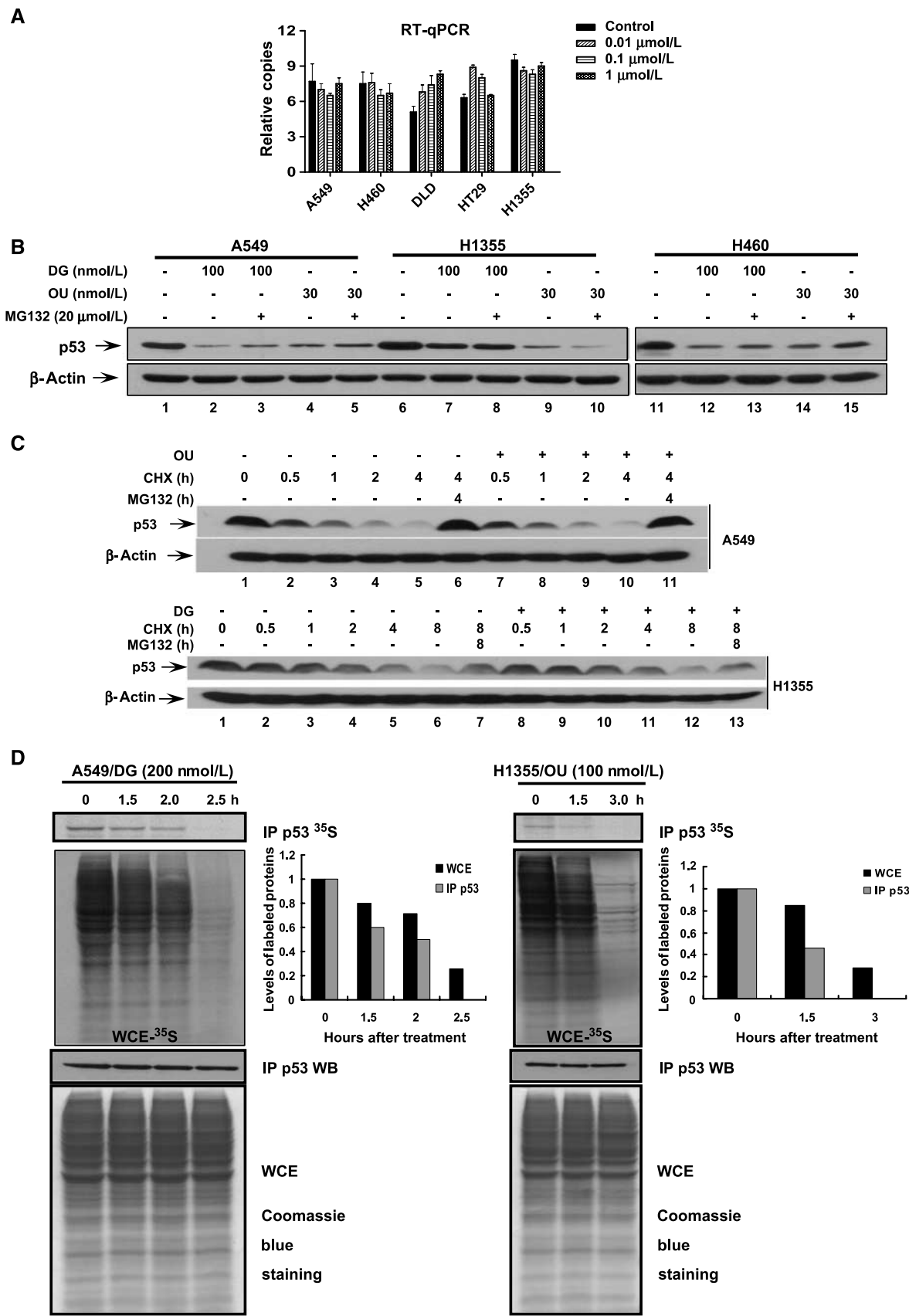
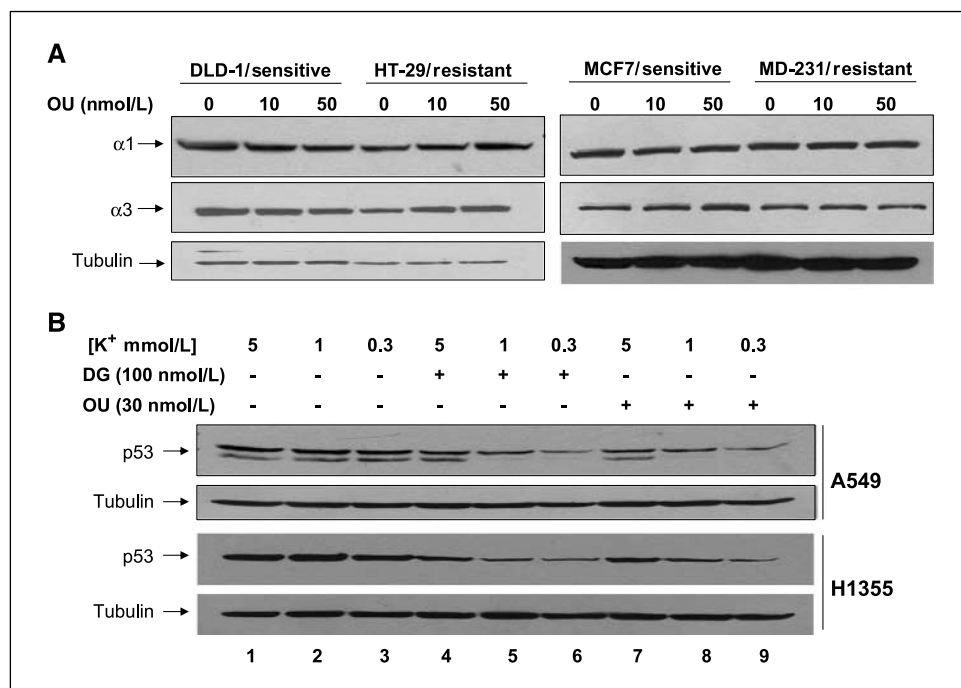


Figure 5. Effect of Na⁺/K⁺-ATPase on p53 reduction by digoxin or ouabain. **A**, the levels of α subunits of the pump did not correlate with cellular sensitivity to p53 reduction: Two sensitive and two resistant colon and breast cancer cell lines were treated with ouabain and the levels of $\alpha 1$ and $\alpha 3$ subunits were measured with Western blotting. **B**, lowering the pump activity enhanced p53 reduction: Cells were cultured in regular medium containing 5 mmol/L potassium or special media with potassium concentration reduced to 1 or 0.3 mmol/L. Cells were left untreated or treated with digoxin or ouabain for 24 h, followed by Western blotting. Note that two bands (p53 and p47) were visualized in A549 cells after prolonged electrophoresis, but both are subjected to reduction by drugs.



translation, or posttranslation, although posttranslational regulation is the most common mechanism for p53 regulation. We first examined if p53 reduction occurred at the mRNA levels. Five cell lines with four sensitive (A549, H460, DLD-1, and H1355) and one resistant (HT29) were treated with digoxin at various doses up to 1 μ mol/L and subjected to RT-qPCR analysis. As shown in Fig. 4A, the mRNA levels were rather consistent with all treatments among all tested lines, regardless of their drug sensitivity or p53 status. Thus, p53 reduction does not occur at the mRNA levels.

We next determined if digoxin or ouabain-induced p53 reduction was due to enhanced degradation. Three sensitive lung cancer lines were treated with digoxin or ouabain, alone or in combination with proteasome inhibitor, MG132 in last 6 hours before cell harvesting. As shown in Fig. 4B, MG132 treatment only slightly, if any, blocked digoxin- or ouabain-induced reduction of p53 levels in A549 and H460 cells (lanes 3 versus 2; 5 versus 4; and 13 versus 12; 15 versus 14), but had no effect on H1355 cells (lanes 7–10). Similar results were obtained when PS341, a potent proteasome inhibitor, was used (data not shown). A minor blockade of p53 reduction by MG132 was also observed in sensitive DLD-1 and MCF-7 cells (data not shown).

We then measured the effect of digoxin or ouabain on p53 protein half-life using A549 or H1355 cells. Cells were treated with cycloheximide to block new protein synthesis in the absence or presence of digoxin or ouabain and harvested at various time points for p53 level measurement by Western blotting. As shown in Fig. 4C, wt p53 has a protein half-life of ~0.5 to 1 hour in

A549 cells (*top*), whereas mutant p53 has a half-life of 2 to 4 hours in H1355 cells (*bottom*). In both cases, however, digoxin or ouabain treatment did not change the p53 half-life. As expected, p53 degradation can be blocked by MG132, independent of drug treatment (lanes 6 and 11 versus 5 and 10; lanes 7 and 13 versus 6 and 12). Taken together, these results indicate that digoxin or ouabain-induced p53 reduction is not due to enhanced degradation.

We finally determined if digoxin or ouabain could inhibit p53 protein synthesis. Two drug sensitive lines A549 and H1355 were used and newly synthesized p53 was measured by ³⁵S-methionine labeling in the presence of MG132 to block p53 degradation. As shown in Fig. 4D, in both lines digoxin or ouabain treatment induced a time-dependent inhibition of *de novo* p53 protein synthesis with a complete elimination of p53 synthesis at 2.5 or 3 hours after treatment, respectively (*top*). The drugs also caused a time-dependent inhibition of overall *de novo* protein synthesis but to less extent (second panel with quantification data shown on the right). In contrast, the same treatment did not change the steady-state levels of p53 (*third panel*), nor the total cellular proteins (*bottom*). The drug also inhibited *de novo* synthesis of p53 in an additional sensitive line, MCF7 (data not shown). Thus, digoxin or ouabain-induced p53 reduction is not due to enhanced degradation, rather due to inhibited protein synthesis.

Cardiac glycosides–induced p53 reduction is independent of the levels of α subunits of Na⁺/K⁺-ATPase, but is enhanced by lowering extracellular K⁺. The cardiac glycosides target for

Figure 4. Reduction of p53 by digoxin or ouabain does not occur at the mRNA levels, but at the protein levels. **A**, lack of p53 mRNA changes upon digoxin treatment: Five cancer cell lines were treated with digoxin for 24 h, followed by RNA isolation and qRT-PCR analysis. The relative p53 mRNA levels were plotted. **B**, proteasome inhibitor failed to rescue p53 reduction: Cells were treated with digoxin or ouabain for 24 h, alone or in combination with MG132 (added in last 6 h), followed by Western blotting. **C**, digoxin or ouabain did not shorten p53 protein half-life: Cells were treated with cycloheximide (CHX) to block new protein synthesis in the absence or presence of digoxin (100 nmol/L) or ouabain (30 nmol/L). Cells were harvested at various time points and subjected to Western blot analysis. **D**, digoxin or ouabain inhibited *de novo* p53 synthesis: Cells were treated with digoxin or ouabain for indicated periods of time with MG132 addition at the last hour. Cells were pulse labeled with ³⁵S-methionine for 5 min, followed by p53 immunoprecipitation (IP) and autoradiography (*top*). Total ³⁵S-methionine incorporation into cellular proteins was assessed by autoradiography (*second panel*). The steady-state levels of p53 was measured by p53 immunoprecipitation, followed by Western blotting (*third panel*), whereas the total amounts of cellular proteins were measured by Coomassie blue staining (*bottom*). WCE, whole cell extracts.

treatment of congestive heart failure is the Na^+/K^+ -ATPase (26). We determine if the levels of Na^+/K^+ -ATPase α subunits are associated with cellular sensitivity to ouabain-induced p53 reduction. As depicted in Fig. 5A, Western blot analyses reveal that both ouabain-sensitive and ouabain-resistant cells express $\alpha 1$ and $\alpha 3$. Apparently, the cellular sensitivity of ouabain-induced p53 reduction did not correlate with the basal levels of Na^+/K^+ -ATPase in these cells. To test if inhibition of Na^+/K^+ -ATPase by means other than cardiac glycosides is sufficient to reduce p53, we lowered extracellular K^+ from 5 mmol/L to 1 and 0.3 mmol/L (27), and measured for p53. As shown in Fig. 5B, reduction of potassium concentration had no effect on the basal levels of p53 (*lanes 1–3*). Interestingly, digoxin- or ouabain-induced p53 reduction was more pronounced progressively, consistent with the fact that lowering extracellular K^+ increases digoxin- and ouabain-binding to the Na^+/K^+ -ATPase. These results clearly showed that digoxin- or ouabain-induced p53 reduction is promoted by the binding of these drugs to the Na^+/K^+ -ATPase.

Cardiac glycosides reduced p53 levels via triggering and activating Src/MAPK signaling pathways. It has been proposed

that the Na^+/K^+ -ATPase is preassembled with its partners in caveolae; the binding of ouabain or digoxin to the pump activates the signalosome to transduce the signals via multiple pathways, including Src, FAK, MAPK, and PI-3K (28–30). We first determined if digoxin or ouabain treatment would activate Src or MAPK in sensitive A549 and H1355 cells. As shown in Fig. 6A, a short treatment of cells with digoxin or ouabain for 5 or 15 minutes caused Src and MAPK activation, as shown by increased phosphorylation at the activation sites (pSrc-Y418 and pERK-T183/Y185). We then determined the effect of Src and FAK inhibition on drug-induced p53 reduction because Src and FAK are two upstream molecules activated upon ouabain- or digoxin-pump binding (29). In both A549 and H1355 cells, a potent Src tyrosine kinase inhibitor PP2 (31) blocked the p53 reduction by digoxin or ouabain in a dose-dependent manner (Fig. 6B, *lanes 3–5* versus *1*; *7–9* versus *6* and *12–15* versus *10*; *17* versus *16*, respectively), whereas the inhibitor itself had no effect on the p53 level (*lane 15*; data not shown). On the other hand, siRNA silencing of FAK in either A549 or H1355 cells had no effect on p53 reduction by digoxin or ouabain (Supplementary Fig. S4). We, therefore, focused

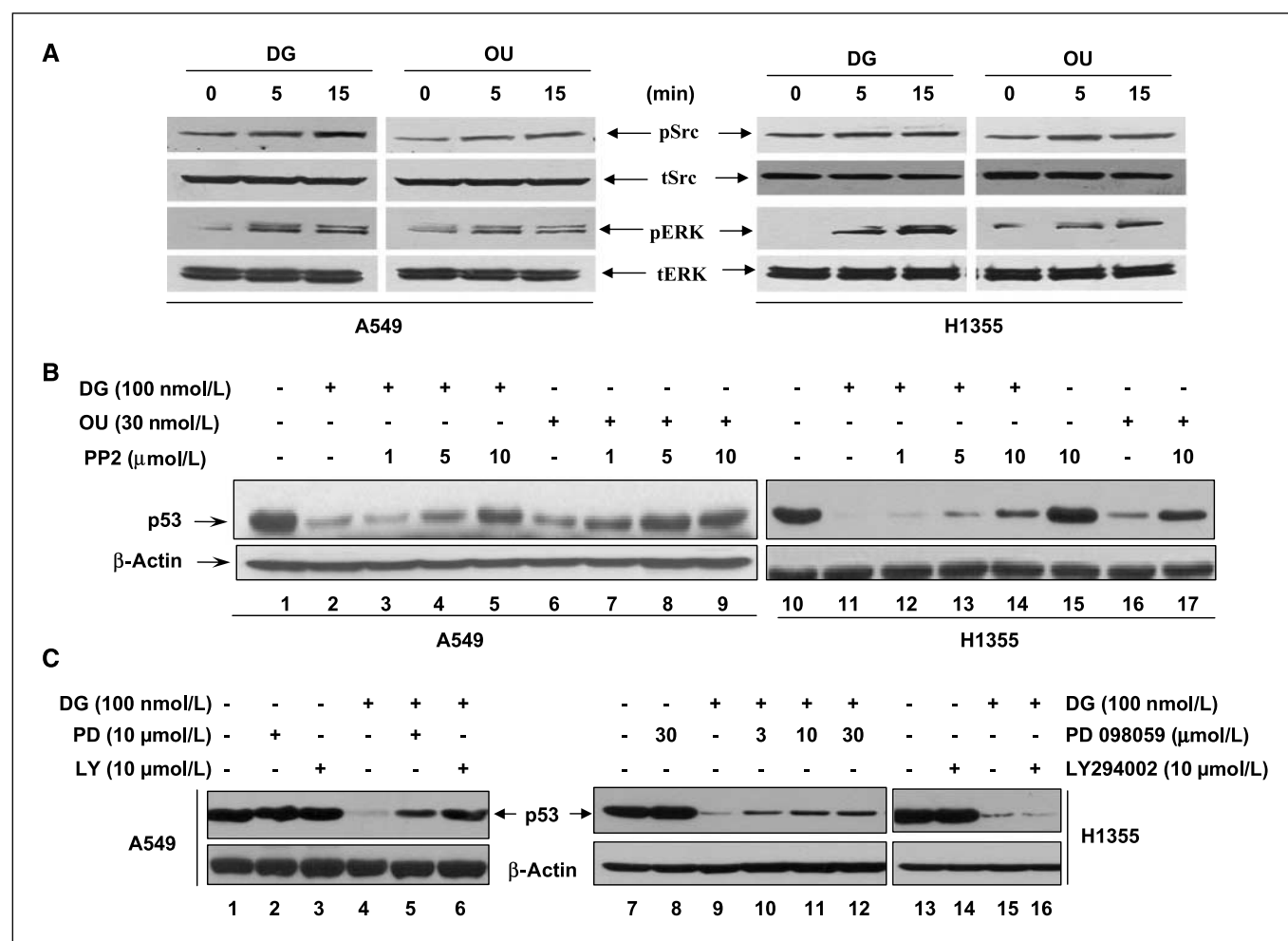


Figure 6. p53 reduction by ouabain or digoxin was mediated by Src/MAPK/PI3K signaling pathways. **A**, activation of Src and MAPK upon ouabain/digoxin exposure. Cells were serum starved for 36 h, followed by exposed to ouabain (30 nmol/L) or digoxin (100 nmol/L) for indicated periods of time, and analyzed by Western blotting. pSrc/pERK, phosphorylated form of Src/ERK; tSrc/tERK, total Src/ERK. **B**, p53 reduction rescued by Src inhibitor PP2. Cells were left untreated or treated with digoxin or ouabain alone or in combination with increasing concentrations of PP2 for 24 h. **C**, p53 reduction rescued by MAPK or PI3K inhibitor. Cells were left untreated or treated with digoxin alone or in combination with MEK inhibitor PD098059 or PI3K inhibitor LY294002 for 24 h. Cell lysates were prepared for Western blotting.

our attention on Src downstream pathways, particularly MAPK and PI3K pathways using specific inhibitors to determine if they blocked p53 reduction. Indeed, whereas PD098059, a MEK inhibitor that blocks MAPK pathway or LY294002, a PI3K inhibitor had no effect on the levels of p53 by drug itself (Fig. 6C, lanes 2 and 3 versus 1 and lanes 8 versus 7 and 14 versus 13), both inhibitors were able to rescue the p53 reduction by digoxin in A549 cells (Fig. 6C, lanes 5 and 6 versus 4). Only MEK inhibitor but not PI3K inhibitor, partially rescued p53 reduction in H1355 cells (Fig. 6C, lanes 10–12 versus 9 and 16 versus 15). Taken together, these results showed that activation of SRC/MAPK/PI3K signaling pathways triggered by the binding of cardiac glycosides to the pump is responsible for p53 reduction. Although the SRC activation mediates p53 reduction in both cell lines, MAPK and PI3K are involved in A549 cells, whereas MAPK, but not PI3K, is partially involved in H1355 cells.

Discussion

Cardiac glycosides are a class of natural products that have been used for medical purposes since ancient time. Three well-known cardiac glycosides, digoxin, ouabain, and digitoxin were used for the treatment of congestive heart failure and atrial fibrillation via binding and inhibiting Na^+/K^+ -ATPase to increase intracellular calcium concentrations (28). In addition to benefit heart failure patients, the drugs were also found to be beneficial to breast cancer patients (32), and were associated with a lower risk for leukemia, lymphoma, as well as kidney and urinary tract cancer (33). Accumulated data in past few years have shown that cardiac glycosides selectively inhibited proliferation and induced apoptosis and autophagy in cancer cells, but not normal cells, suggesting their utility in anticancer therapy (ref. 34, and for review, see ref. 28). Mechanistically, digoxin or ouabain inhibited catalytic activity of topoisomerase II (35) and stabilized DNA-topoisomerase II complexes to suppress growth. Digoxin, ouabain, or other cardiac glycosides up-regulated death receptor 4 and 5 to sensitize lung cancer cells to apoptosis induced by Apo2L/TRAIL (36). Digoxin or ouabain also remarkably inhibit protein synthesis of HIF-1 α to block HIF1 transcription factor activity and to inhibit tumor cell growth both *in vitro* and *in vivo* (37). Furthermore, digoxin or ouabain could modulate signaling pathways of MAPK/AKT, PKC/ activator protein, nuclear factor- κ B, and reactive oxygen species to regulate cell growth and survival (for reviews, see refs. 26, 28). Finally, globe level reduction of protein synthesis upon drug exposure, as shown in our study (Fig. 4D), could also contribute to their cytotoxicity. However, the role of p53 in the action of cardiac glycosides is totally unknown, although a recent study showed an observation that ouabain slightly reduced p53 level in a breast cancer line, MDA-MB-435s, without providing any mechanistic insight (38).

Digoxin was identified in our chemical library screening for the drugs that selectively kill cancer cells with mutant p53 via synthetic lethal mechanism (21). Subsequent analysis serendipitously found that the drug effectively reduces p53 levels in a cell line-dependent, but p53 status-independent manner. It is well-known that p53 is a short-lived protein whose expression was regulated mainly at the posttranslational levels, including phosphorylation, acetylation, ubiquitination, sumoylation, and methylation (39–41). Recently, p53 is shown to be regulated at the translational level on protein synthesis by ribosomal protein

L26 (42), mammalian target of rapamycin (43) or Mdm2 (44). Interestingly, Mdm2 could either stimulate p53 synthesis via a direct binding to p53 mRNA (44) or inhibit p53 synthesis by promoting the degradation of L26 (45). We found that the drug-induced p53 reduction did not occur at the mRNA level, as shown by RT-qPCR analysis, but at the protein level. Failure to rescue drug-induced p53 reduction by proteasome inhibitors and failure of drugs to shorten the p53 protein half-life indicate that p53 reduction by the drugs is not due to enhanced p53 degradation. The drug inhibition on metabolic labeling of newly synthesized p53 suggests that the change occur at the level of protein synthesis. Thus, digoxin and ouabain are potent inhibitors of p53 protein synthesis.

What is then the mechanism by which digoxin or ouabain inhibits the *de novo* p53 protein synthesis? Although the details are still unknown at the present time, it seems to involve Src/MAPK signaling pathways, triggered and activated by ouabain/digoxin binding to Na^+/K^+ -ATPase because (a) ouabain or digoxin activates Src and MAPK, (b) their inhibitors are able to abrogate the ouabain/digoxin-induced p53 reduction, whereas (c) inhibition of Na^+/K^+ -ATPase activity by lowering extracellular K^+ has no effect. Thus, a simplest explanation for these observations is that upon digoxin or ouabain binding to Na^+/K^+ -ATPase, Src/MAPK signaling pathways are activated particularly in drug-sensitive cancer cells, leading to activation of their downstream effectors, eventually the inhibition of p53 protein synthesis. Future effort will be directed (a) to characterize which type of p53 synthesis, cap dependent and/or cap independent, also known as internal ribosome entry site element dependent (46), is actually inhibited by cardiac glycosides and (b) to elucidate the detailed mechanism of action.

Another interesting observation reported here is that cardiac glycosides, digoxin and ouabain, are potent cytotoxic agents in a panel of tumor cell lines with IC_{50} s ranging from 50 to 100 nmol/L. However, the cytotoxicity is tumor cell line dependent, but independent of wt p53, dissociating cancer cell killing from wt p53 elimination. Although wt p53 induces growth arrest and apoptosis in most cases (1, 6, 7), and acts as a survival protein in some particular cases (for review, see ref. 47), it is unlikely that wt p53 plays any significant role in digoxin or ouabain-induced cell killing in few cancer cell lines tested. Nevertheless, the fact that cardiac glycosides are inactive against wt p53 in normal cells, but potentially active in elimination of mutant p53 in some cancer cells, suggests that these drugs could have utility in the treatment of human cancer harboring a gain-of-function p53 mutant (48, 49).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000;408:307–10.
2. Chipuk JE, Kuwana T, Bouchier-Hayes L, et al. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 2004;303:1010–4.
3. Mihara M, Erster S, Zaika A, et al. p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* 2003;11:577–90.
4. Bouchet BP, de Fromental CC, Puisieux A, Galmarini CM. p53 as a target for anti-cancer drug development. *Crit Rev Oncol Hematol* 2006;58:190–207.
5. Sun Y. p53 and its downstream proteins as molecular targets of cancer. *Mol Carcinog* 2006;45:409–15.
6. Bassett EA, Wang W, Rastinejad F, El-Deiry WS. Structural and functional basis for therapeutic modulation of p53 signaling. *Clin Cancer Res* 2008;14:6376–86.
7. Vazquez A, Bond EE, Levine AJ, Bond GL. The genetics of the p53 pathway, apoptosis and cancer therapy. *Nat Rev Drug Discov* 2008;7:979–87.
8. Foster BA, Coffey HA, Morin MJ, Rastinejad F. Pharmacological rescue of mutant p53 conformation and function. *Science* 1999;286:2507–10.
9. Bykov VJ, Issaeva N, Shilov A, et al. Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound. *Nat Med* 2002;8:282–8.
10. Peng Y, Li C, Chen L, Sebt S, Chen J. Rescue of mutant p53 transcription function by ellipticine. *Oncogene* 2003;22:4478–87.
11. Yang Y, Ludwig RL, Jensen JP, et al. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. *Cancer Cell* 2005;7:547–59.
12. Vassilev LT, Vu BT, Graves B, et al. *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 2004;303:844–8.
13. Issaeva N, Bozko P, Enge M, et al. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med* 2004;10:1321–8.
14. Shangary S, Qin D, McEachern D, et al. Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci U S A* 2008;105:3933–8.
15. Shangary S, Ding K, Qiu S, et al. Reactivation of p53 by a specific MDM2 antagonist (MI-43) leads to p21-mediated cell cycle arrest and selective cell death in colon cancer. *Mol Cancer Ther* 2008;7:1533–42.
16. Sun SH, Zheng M, Ding K, Wang S, Sun Y. A small molecule that disrupts Mdm2–53 binding activates p53, induces apoptosis, and sensitizes lung cancer cells to chemotherapy. *Cancer Biol Ther* 2008;7:845–52.
17. Komarov PG, Komarova EA, Kondratov RV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 1999;285:1733–7.
18. Strom E, Sathe S, Komarov PG, et al. Small-molecule inhibitor of p53 binding to mitochondria protects mice from γ radiation. *Nat Chem Biol* 2006;2:474–9.
19. Blagosklonny MV, Toretsky J, Neckers L. Geldanamycin selectively destabilizes and conformationally alters mutated p53. *Oncogene* 1995;11:933–9.
20. Blagosklonny MV, Trostel S, Kayastha G, et al. Depletion of mutant p53 and cytotoxicity of histone deacetylase inhibitors. *Cancer Res* 2005;65:7386–92.
21. Kaelin WG. The concept of synthetic lethality in the context of anticancer therapy. *Nat Rev Cancer* 2005;5:689–98.
22. Zheng M, Morgan-Lappe SE, Yang J, et al. Growth inhibition and radiosensitization of glioblastoma and lung cancer cells by small interfering RNA silencing of tumor necrosis factor receptor-associated factor 2. *Cancer Res* 2008;68:7570–8.
23. Bowen JW, McDonough A. Pretranslational regulation of Na-K-ATPase in cultured canine kidney cells by low K⁺. *Am J Physiol* 1987;252:C179–89.
24. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1 α . *Nature* 1998;392:405–8.
25. Robinson M, Jiang P, Cui J, et al. Global Genechip profiling to identify genes responsive to p53-induced growth arrest and apoptosis in human lung carcinomas. *Cancer Biol Ther* 2003;2:406–15.
26. Schoner W, Scheiner-Bobis G. Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth. *Am J Physiol Cell Physiol* 2007;293:C509–36.
27. Tepperman K, Millette LA, Johnson CL, Jewell-Motz EA, Lingrel JB, Wallick ET. Mutational analysis of Glu-327 of Na(+)-K(+)-ATPase reveals stimulation of 86Rb⁺ uptake by external K⁺. *Am J Physiol* 1997;273:C2065–79.
28. Newman RA, Yang P, Pawlus AD, Block KI. Cardiac glycosides as novel cancer therapeutic agents. *Mol Interv* 2008;8:36–49.
29. Xie Z, Cai T. Na⁺-K⁺-ATPase-mediated signal transduction: from protein interaction to cellular function. *Mol Interv* 2003;3:157–68.
30. Liang M, Cai T, Tian J, Qu W, Xie ZJ. Functional characterization of Src-interacting Na/K-ATPase using RNA interference assay. *J Biol Chem* 2006;281:19709–19.
31. Hanke JH, Gardner JR, Dow RL, et al. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 1996;271:695–701.
32. Stenkvist B, Bengtsson E, Eriksson O, Holmquist J, Nordin B, Westman-Naeser S. Cardiac glycosides and breast cancer. *Lancet* 1979;1:563.
33. Haux J, Klepp O, Spigset O, Tretli S. Digitoxin medication and cancer: case control and internal dose-response studies. *BMC Cancer* 2001;1:11.
34. Newman RA, Kondo Y, Yokoyama T, et al. Autophagic cell death of human pancreatic tumor cells mediated by oleandrin, a lipid-soluble cardiac glycoside. *Integr Cancer Ther* 2007;6:354–64.
35. Bielawski K, Winnicka K, Bielawska A. Inhibition of DNA topoisomerases I and II, and growth inhibition of breast cancer MCF-7 cells by ouabain, digoxin and proscillaridin A. *Biol Pharm Bull* 2006;29:1493–7.
36. Frese S, Frese-Schaper M, Andres AC, Miescher D, Zumkehr B, Schmid RA. Cardiac glycosides initiate Apo2L/TRAIL-induced apoptosis in non-small cell lung cancer cells by up-regulation of death receptors 4 and 5. *Cancer Res* 2006;66:5867–74.
37. Zhang H, Qian DZ, Tan YS, et al. Digoxin and other cardiac glycosides inhibit HIF-1 α synthesis and block tumor growth. *Proc Natl Acad Sci U S A* 2008;105:19579–86.
38. Kometiani P, Liu L, Askari A. Digitalis-induced signaling by Na⁺/K⁺-ATPase in human breast cancer cells. *Mol Pharmacol* 2005;67:929–36.
39. Bode AM, Dong Z. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer* 2004;4:793–805.
40. Brooks CL, Gu W. Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Curr Opin Cell Biol* 2003;15:164–71.
41. Scoumanne A, Chen X. Protein methylation: a new mechanism of p53 tumor suppressor regulation. *Histol Histopathol* 2008;23:1143–9.
42. Takagi M, Absalon MJ, McLure KG, Kastan MB. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* 2005;123:49–63.
43. Lee CH, Inoki K, Korbowniczek M, et al. Constitutive mTOR activation in TSC mutants sensitizes cells to energy starvation and genomic damage via p53. *EMBO J* 2007;26:4812–23.
44. Candeias MM, Malbert-Colas L, Powell DJ, et al. p53 mRNA controls p53 activity by managing Mdm2 functions. *Nat Cell Biol* 2008;10:1098–1105.
45. Ofir-Rosenfeld Y, Boggs K, Michael D, Kastan MB, Oren M. Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. *Mol Cell* 2008;32:180–9.
46. Halaby MJ, Yang DQ. p53 translational control: a new facet of p53 regulation and its implication for tumorigenesis and cancer therapeutics. *Gene* 2007;395:1–7.
47. Janicke RU, Sohn D, Schulze-Osthoff K. The dark side of a tumor suppressor: anti-apoptotic p53. *Cell Death Differ* 2008;15:959–76.
48. Di Agostino S, Strano S, Emiliozzi V, et al. Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. *Cancer Cell* 2006;10:191–202.
49. Song H, Hollstein M, Xu Y. p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. *Nat Cell Biol* 2007;9:573–80.
50. Peng X, Wu X, Druso JE, et al. Cardiac developmental defects and eccentric right ventricular hypertrophy in cardiomyocyte focal adhesion kinase (FAK) conditional knockout mice. *Proc Natl Acad Sci U S A* 2008;105:6638–43.